Remarks

Reconsideration and withdrawal of the objection to claim 1 and the rejections of the claims, in view of the amendments and remarks herein, is respectfully requested. Claim 1 is amended. The amendments are intended to advance the application and are not intended to concede to the correctness of the Examiner's position or to prejudice the prosecution of the claims prior to amendment, which claims are present in a continuation of the present application. Claims 1, 3-6, 8-14, and 16-36 are now pending in this application.

The Examiner objected to the phrase "sialic acid residues" in line 5 of claim 1, however, line 5 recites "sialic acid containing residues" and "sialic acid-containing host cell receptors." As the objected-to phrase does not appear in line 5 of claim 1, further clarification is requested.

The Examiner rejected claims 1, 3-6, 8-11, and 32-35 under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. In particular, the Examiner asserts that the following in claim 1 are inconsistent "cell comprising decreased levels of terminal sialic acid-containing host cell receptors for influenza virus relative to a corresponding wild-type cell" and "cell has decreased levels of *N*-acetylneuraminic acid and/or decreased levels of *N*-glycolylneuraminic acid relative to the corresponding wild-type cell," and "sialic acid-containing host cell receptors" and "terminal sialic acid-containing host cell receptors." The Examiner also asserts it is unclear whether the mutant cell has decreased levels of terminal sialic acid containing host cell receptors or decreased levels of *N*-acetylneuraminic acid and/or *N*-glycolylneuraminic acid. These rejections, as they may be maintained with respect to the pending claims, are respectfully traversed.

"Sialic acid" is a general term for a nine carbon, amino containing monosaccharide that typically forms the terminal residues of cell surface oligosaccharides (see www.bme.jhu.edu/~kjyarema/monosaccharides). There are over 50 natural forms of sialic acid (www.bme.jhu.edu/~kjyarema/monosaccharides). Two examples of sialic acid are *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid, and it is common that they are appended to galactose or *N*-acetylgalactosamine by a $\alpha(2,3)$ or $\alpha(2,6)$ glycosidic linkage (www.bme.jhu.edu/~kjyarema/monosaccharides). It is well-known that influenza virus binds

sialic acid containing residues (see page 1, lines 17-21 of Applicant's specification). Thus, the language in claim 1 would be clear to one of skill in the art.

Accordingly, withdrawal of the 35 U.S.C. § 112, second paragraph, rejections is respectfully requested.

The Examiner rejected claims 1, 3-4, 8, 32, and 34-36 under 35 U.S.C. § 102(b) as being anticipated by Martin et al. (Virology, 241:101 (1998)) or Brandli et al. (J. Biol. Chem., 263:16283 (1988)) as evidenced by Doyle et al. (U.S. published application No. 20040132164) and Ito et al. (J. Virol., 71:3357 (1997)). The Examiner also rejected claims 1, 3, 9, 33, and 36 under 35 U.S.C. § 102(b) as being anticipated by Tazikawa et al. (JP 407203958A) as evidenced by Doyle et al. These rejections, as they may be maintained with respect to the pending claims, are respectfully traversed.

Martin et al. disclose that influenza virus HA proteins with substitutions in the receptor binding site can affect the ability of HA to bind to human erythrocytes, presumably due to the reduced affinity of mutant HA for sialic acid (pages 105-106). It is disclosed that four transfectant viruses with mutant HAs were able to infect MDCK cells and embryonated chicken eggs with efficiencies comparable to wild-type (page 106), although the infectivity of one of the transfectant viruses on a mutant ricin-resistant MDCK cell ("MDCK RCA^r") was greatly reduced compared to wild-type MDCK cells. It is further disclosed that MDCK RCA^r cells have a 70 to 75% reduction in cell surface sialic acid (citing to Brandli et al., 1988), and that these cells may produce reduced virus yields (citing Green et al., J. Cell. Biol., 89:230 (1981)).

Brandli et al. disclose that a ricin-resistant MDCK cell line (MDCKII-RCA^r) and wild-type cells bind wheat germ agglutinin (specific for *N*-acetylglucosamine and *N*-acetylneuraminic acid), conconavalin A (specific for mannose) and *H. pomatia* agglutinin (*N*-acetylgalactosamine), which binding was unaffected by exogalactosylation (page 16286). It is further disclosed that wild-type cells did not contain significant amounts of *N*-acetylglucosamine (assessed by *B. simplicifolia* agglutinin binding) while mutant cells bound *B. simplicifolia* agglutinin, which could be eliminated by exogalactosylation. In contrast to wild-type cells, it is disclosed that mutant cells did not bind peanut lectin (specific for terminal galactose linked to *N*-acetylgalactosamine). While mutant cells had decreased binding to (70 to 75%) *Limax flavus* agglutinin (LFA, a lectin which binds sialyl residues in a non-glycosidic linkage specific manner,

see Cross et al., J. Biol. Chem., 278:4112 (2003), of record) (pages 16287-8 of Brandli et al.), Brandli et al. conclude that MDCKII-RCA^r cells are deficient in the addition of galactose residues to *N*- and *O*-linked glycans (page 16286).

Ito et al. incubated frozen sections of avian allantoic cells, avian amniotic cells, and MDCK cells with digoxigenin labeled *Maakia amurensis* (MAA) lectin or *Sambucus nigra* (SNA) lectin to characterize the lectin binding specificity of those cells. Those cells were not grown in the presence of lectin, e.g., to select for cells that were to resistant to lectin growth inhibition.

Doyle et al. describe compositions and methods for enzymatic reduction of adhesion by microorganisms to cells, tissues, extracellular matrix teeth and/or dental prosthesis. Doyle et al. mention that polyphenol oxidase and the asparaginase are effective in reducing influenza A virus attachment to sialic acid containing red blood cells [see paragraph 320].

With regard to the ricin-resistant cells in Martin et al. and Brandli et al., the Examiner is requested to consider that ricin is known to bind galactose (see, e.g., Gottlieb et al., J. Biol. Chem., 251:7761 (1976)) and in particular bind β1,4-linked galactose (Lord et al., Toxicol. Rev., 22:53 (2003) and Hartley et al., Biochem. Biophys. Acta, 1701:1 (2004)) (a copy of each is enclosed herewith).

In contrast, Applicant's mutant cell was selected for resistance to *Maakia amurensis* lectin, which binds sialic acid linked to galactose by α(2,3) linkages, or *Sambucus nigra* lectin, which binds sialic acid linked to galactose by α(2,6) linkages. Therefore, Martin et al. and Brandl et al. do not teach Applicant's mutant cell.

The abstract for JP407203958A provided by the Examiner discloses that UV rays, X-rays and an alkylating agent may be used to mutate a CHO cell and mutants selected that have reduced "productivity of *N*-glycolylneuraminic acid". The abstract discloses that one resulting mutant strain had "5/14 productivity" of *N*-glycolylneuraminic acid and "≤ 1/5 hydroxylase activity" of CMP-*N*-acetylneuraminic acid as compared to the parent CHO strain.

A copy of a translation of JP407203958A is enclosed herewith. JP407203958A discloses that CHO cells were mutagenized with a chemical mutagen, then selected for resistance to complement mediated anti-GM3 antibody-induced lysis. 9 strains with reduced GM3 expression were analyzed for GM3 and sugar synthesis, and one of the strains, 1A51, was further

analyzed. 1A51 was shown to have reduced *N*-glycolylneuraminic acid (NeuGc) content relative to the parent CHO strain (5% versus 14.2% NeuGc content, respectively) as a result of reduced activity of CMP-NeuAc hydoxylase, which is disclosed as hydoxylating CMP-NeuAc to generate NeuGc. NeuAc content in 1A51 cells is not substantially different than in the parent strain (see Figure 2).

As disclosed in Applicant's specification, CHO cells do not support efficient influenza virus replication (page 24, lines 14-16) (see claim 1). Thus, JP407203958A does not disclose Applicant's mutant cell.

Accordingly, withdrawal of the § 102(b) rejections is respectfully requested.

CONCLUSION

Applicant respectfully submits that the claims are in condition for allowance, and notification to that effect is earnestly requested. The Examiner is invited to telephone Applicant's attorney at (612) 373-6959 to facilitate prosecution of this application.

If necessary, please charge any additional fees or credit overpayment to Deposit Account No. 19-0743.

Respectfully submitted,

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Date January 24, 2006 By _____
Janet E. Embretson
Reg. No. 39,665

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1: J Biol Chem. 1976 Dec 25;251(24):7761-8. Related Articles, Links

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Isolation and characterization of two mouse L cell lines resistant to the toxic lectin ricin.

Gottlieb C, Kornfeld S.

Two variant mouse L cell lines (termed CL 3 and CL 6) have been selected for resistant to ricin, a galactose-binding lectin with potent cytotoxic activity. The resistant lines exhibit a 50 to 70% decrease in ricin binding and a 300- to 500-fold increase in resistance to the toxic effects of ricin. Crude membrane preparations of CL 3 cells have increased sialic acid content (200% of control), while the galactose, mannose, and hexosamine content is within normal limits. Both the glycoproteins and glycolipids of CL 3 cells have increased sialic acid, with the GM3:lactosylceramide ratios for parent L and CL 3 cells being 0.29 and 1.5, respectively. In contrast, the membranes of CL 6 cells have a decrease in sialic acid, galactose, and hexosamine content with mannose being normal. Both cell lines have specific alterations in glycosyltransferase activities which can account for the observed membrane sugar changes. CL 3 cells have increased CMP-sialic acid:glycoprotein sialyltransferase and GM3 synthetase activities, while CL 6 cells have decrease UDP-GlcNAc:glycoproteinN-acetylglucosaminyltransferase and DPU-galactose:glycoprotein galactosyltransferase activities. The increased sialic acid content of CL 3 cells serves to mask ricin binding sites, since neuraminidase treatment of this cell line restores ricin binding to essentially normal levels. However, the fact that neuraminidase-treated CL 3 cells are still 45-fold resistant to ricin indicates that either a special class of productive ricin binding sites is not being exposed or that the cell line has a second mechanism for ricin resistance.

PMID: 1002711 [PubMed - indexed for MEDLINE]

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1: Toxicol Rev. 2003;22(1):53-64.

Ricin. Mechanisms of cytotoxicity.

Lord MJ, Jolliffe NA, Marsden CJ, Pateman CS, Smith DC, Spooner RA, Watson PD, Roberts LM.

Department of Biological Sciences, University of Warwick, Coventry, UK.

Ricin is a heterodimeric protein produced in the seeds of the castor oil plant (Ricinus communis). It is exquisitely potent to mammalian cells, being able to fatally disrupt protein synthesis by attacking the Achilles heel of the ribosome. For this enzyme to reach its substrate, it must not only negotiate the endomembrane system but it must also cross an internal membrane and avoid complete degradation without compromising its activity in any way. Cell entry by ricin involves a series of steps: (i) binding, via the ricin B chain (RTB), to a range of cell surface glycolipids or glycoproteins having beta-1,4-linked galactose residues; (ii) uptake into the cell by endocytosis; (iii) entry of the toxin into early endosomes; (iv) transfer, by vesicular transport, of ricin from early endosomes to the trans-Golgi network; (v) retrograde vesicular transport through the Golgi complex to reach the endoplasmic reticulum; (vi) reduction of the disulphide bond connecting the ricin A chain (RTA) and the RTB; (vii) partial unfolding of the RTA to render it translocationally-competent to cross the endoplasmic reticulum (ER) membrane via the Sec61p translocon in a manner similar to that followed by misfolded ER proteins that, once recognised, are targeted to the ER-associated protein degradation (ERAD) machinery; (viii) avoiding, at least in part, ubiquitination that would lead to rapid degradation by cytosolic proteasomes immediately after membrane translocation when it is still partially unfolded; (ix) refolding into its protease-resistant, biologically active conformation; and (x) interaction with the ribosome to catalyse the depurination reaction. It is clear that ricin can take advantage of many target cell molecules, pathways and processes. It has been reported that a single molecule of ricin reaching the cytosol can kill that cell as a consequence of protein synthesis inhibition. The ready availability of ricin, coupled to its extreme potency when administered intravenously or if inhaled, has identified this protein toxin as a potential biological warfare agent. Therapeutically, its cytotoxicity has encouraged the use of ricin in 'magic

bullets' to specifically target and destroy cancer cells, and the unusual intracellular trafficking properties of ricin potentially permit its development as a vaccine vector. Combining our understanding of the ricin structure with ways to cripple its unwanted properties (its enzymatic activity and promotion of vascular leak whilst retaining protein stability and important immunodominant epitopes), will also be crucial in the development of a long awaited protective vaccine against this toxin.

Publication Types:

- Review

PMID: 14579547 [PubMed - indexed for MEDLINE]

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1: Biochim Biophys Acta. 2004 Sep 1;1701(1-2):1-14. Related Articles, Links

ELSEVIER FULL-TEXT ARTICLE

Cytotoxic ribosome-inactivating lectins from plants.

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A class of heterodimeric plant proteins consisting of a carbohydrate-binding B-chain and an enzymatic A-chain which act on ribosomes to inhibit protein synthesis are amongst the most toxic substances known. The best known example of such a toxic lectin is ricin, produced by the seeds of the castor oil plant, Ricinnus communis. For ricin to reach its substrate in the cytosol, it must be endocytosed, transported through the endomembrane system to reach the compartment from which it is translocated into the cytosol, and there avoid degradation making it possible for a few molecules to inactivate a large proportion of the ribosomes and hence kill the cell. Cell entry by ricin involves the following steps: (i) binding to cell-surface glycolipids and glycoproteins bearing beta-1,4-linked galactose residues through the lectin activity of the B-chain (RTB); (ii) uptake by endocytosis and entry into early endosomes; (iii) transfer by vesicular transport to the trans-Golgi network; (iv) retrograde vesicular transport through the Golgi complex and into the endoplasmic reticulum (ER); (v) reduction of the disulfide bond connecting the A- and B-chains; (vi) a partial unfolding of the A-chain (RTA) to enable it to translocate across the ER membrane via the Sec61p translocon using the pathway normally followed by misfolded ER proteins for targeting to the ER-associated degradation (ERAD) machinery; (vi) refolding in the cytosol into a protease-resistant, enzymatically active structure; (vii) interaction with the sarcin-ricin domain (SRD) of the large ribosome subunit RNA followed by cleavage of a single N-glycosidic bond in the RNA to generate a depurinated, inactive ribosome. In addition to the highly specific action on ribosomes, ricin and related ribosome-inactivating proteins (RIPs) have a less specific action in vitro on DNA and RNA substrates releasing multiple adenine, and in some instances, guanine residues. This polynucleotide:adenosine glycosidase activity has been implicated in the general antiviral, and specifically, the anti HIV-1 activity

of several single-chain RIPs which are homologous to the A-chains of the heterodimeric lectins. However, in the absence of clear cause and effect evidence in vivo, such claims should be regarded with caution.

Publication Types:

- Review
- Review, Tutorial

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Japanese Kokai Patent Application No. Hei 7[1995]-203958

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MUTANT CHINESE HAMSTER OVARY CELL STRAINS

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[There are no amendments to this patent.]

Abstract

Constitution

Mutant Chinese hamster ovary (CHO) cell strains with reduced N-glycolylneuraminic acid production, and genetic recombination-based glycoprotein production methods using said mutant strains as host cells.

Effect

Since these mutant CHO cell strains have reduced NeuGc production, glycoproteins manufactured by recombinant gene technology utilizing these as host cells contain almost no NeuGc. Consequently, these mutant strains are useful as host cells for manufacturing glycoproteins with no H-D antigenicity.

Claims

1. Mutant Chinese hamster ovary cell strains with reduced N-glycolylneuraminic acid production.

2. Mutant strains described in Claim 1 wherein the N-glycolylneuraminic acid production is less than 1/2 of non-mutant strains.

3. Mutant strains described in Claim 1 or 2 wherein the activity of cytidine-5'-monophosphate N-acetylneuraminic acid hydroxylase is less than 1/5 of non-mutant strains.

4. Mutant strains described in Claim 1, 2 or 3 that are obtained by treating Chinese hamster ovary cells to induce mutations.

5. Methods for manufacturing glycoproteins characterized by the fact that recombinant vectors having genes that code for glycoproteins are introduced in the mutant strains described in Claim 1 and the transformants obtained are cultured.

Detailed explanation of the invention

[0001]

Industrial application field

The present invention pertains to mutant Chinese hamster ovary cell strains (abbreviated CHO below). More specifically, it pertains to mutant CHO cell strains that are useful as host cells for recombinant gene technology, and their use.

[0002]

Prior art

Attempts to produce physiologically active substances derived from humans that are present in trace amounts in the body in large quantities using recombinant gene technology and to adapt these to pharmaceutical products are actively being made. In said recombinant gene technology, *E. coli* is the most widely used host cell the target of introducing recombinant vectors because of ease of handling, proliferation and culture. However, since physiologically active substances originally derived from humans are often glycoproteins, when they are pressed in microbes such as *E. coli*, sugar chains are often not attached. Therefore, in recent years, there has been a trend toward production using mammalian cells instead of *E. coli* as host cells to obtain target substances in forms that are closer to human-derived physiologically active substances. CHO cells are widely used as said mammalian host cells.

[0003]

Problems to be solved by the invention

However, because there is species specificity in sugar chain structures, it has been pointed out that, when using non-human cells such as CHO cells, products may be immunogens to humans due to sugar chains being attached that differ from those of humans [Hokke, C.H. et al.: FEBS Letters, 275: 9-14 (1990)].

[0004]

CHO cells are no exception. The sialic acid composition differs between human and CHO cells. That is, because CHO cells have N-glycolylneuramic acid while humans normally do not have N-glycolylneuramic acid, glycoproteins produced using CHO cells are ones to which compound sugars, including N-glycolylneuramic acid, have been attached. These N-glycolylneuramic acid-containing compound sugars are strongly immunogenic to humans and have been known for many years as the H-D antigen (Hanganutziu-Deicher antigen). Glycoproteins produced in this manner using CHO cells have the drawback that there was a high possibility that they will be immunogenic due to the H-D antigen.

[0005]

Consequently, the purpose of this invention is to present a novel mutant CHO cell strain that can be used in producing physiologically active substances that are not immunogenic due to the H-D antigen, and methods for producing glycoproteins using said mutant strain as host cells.

[0006]

Means to solve the problem

Upon treating CHO cells to induce mutations and studying the sialic acid composition, sugar-related enzyme activities, etc., of the mutant strains obtained, the present inventors succeeded in harvesting novel mutant strains with reduced N-glycolylneuramic acid-producing ability and completed this invention.

[0007]

That is, this invention pertains to mutant CHO cell strains with reduced N-glycolylneuramic acid production.

[0008]

This invention also pertains to glycoprotein production methods characterized by the fact that recombinant vectors having genes that code for glycoproteins are introduced in these mutant CHO cell strains with reduced N-glycolylneuraminic acid production and the transformants obtained are cultured.

[0009]

The mutant strains of this invention have the same properties as the usual CHO cells aside from the fact that production of N-glycolylneuraminic acid (abbreviated NeuGc below) is reduced compared to non-mutant strains. It is preferable that the NeuGc-production of the mutant strains of this invention is less than 1/2 of non-mutant strains. It is preferable that the reduction in NeuGc production of the mutant strains of this invention arises from the fact that cytidine-5'-monophosphate N-acetylneuraminic acid hydroxylase activity is reduced compared to non-mutant strains and conversion from cytidine-5'-monophosphate N-acetylneuraminic acid (CMP-NeuAc) to cytidine-5'-monophosphate N-glycolylneuraminic acid (CMP-NeuGc) does not occur, and that said hydroxylase activity is reduced to less than 1/5 of non-mutant strains.

[0010]

The mutant strains of this invention are produced by treating CHO cells to induce mutations and selecting strains wherein NeuGc production is reduced.

[0011]

The CHO cells used are not particularly restricted as long as they are ones that have been established as normally cultured cell lines. For examples, CHO-K1 cells, CHO-AA8 cells, CHO-EM9 cells, CHO-Pro^{-5} cells, CHO/dhFr^{-} cells, etc., can be cited.

[0012]

Treatments to induce mutations can be treatments that use either physical or chemical mutagens. For physical mutagens, ultraviolet rays, X-rays, etc., can be cited. For chemical mutagens, alkylating agents such as ethylmethanesulfonate (EMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ethylnitrosourea (ENU), etc.; base analogues such as bromodeoxyuridine (BrdUrd), N^4-aminocytidine, etc.; and intercalating ICR [Institute for Cancer Research] compounds, etc., can be cited. Of these, chemical mutagens, particularly alkylating agents and especially EMS, are favorable.

[0013]

In the case of chemical mutagenesis, for example, CHO cell mutagenesis is performed by culturing 10^6 to 10^9 logarithmic growth phase CHO cells in a medium in which CHO cells can proliferate at 25-40°C for 0.5-30 h in the presence of a chemical mutagen in amounts at which the survival rate is 10-50%. The favored media used are Ham's F-12 medium containing 5-10% fetal calf serum, etc., D-MEM medium, RPMI 1640 medium, or serum-free medium. When EMS is used as mutagen, mutagenesis by culturing in medium containing 100-1000 μg/mL of EMS at 25-40°C for 5-30 h is favorable.

[0014]

To screen the mutagen-treated CHO cell groups for the mutant strains of this invention, methods that measure NeuGlc content directly by sialic acid analysis, methods that measure CMP-NeuAc hydroxylase activity, methods that measure the presence or absence of H-D antigenicity, etc., can be cited. Appropriately combining a method for selecting cells in which GM3 amounts on the cell surface are reduced using anti-[ganglisside]GM3 (NeuAcα2 → 3Galβ1 → 4Glcβ1 → 1Cer) antibodies and a method for in situ glycolipid assay with sialic acid analysis and measurement of CMP-NeuAc hydroxylase activity is favorable. After screening by combining a method for selecting cells in which GM3 amounts on the cell surface are reduced using anti-GM3 antibodies with an in situ glycolipid assay, it is particularly favorable to confirm the properties of the mutant strain by sialic acid analysis or measuring CMP-NeuAc hydroxylase activity.

[0015]

Strain 1A51, which is 1 example of a mutant strain of this invention thus obtained, is a novel strain and, compared to CHO-K1 cells, the NeuGc content (production) is about 5/14. CMP-NeuAc hydroxylase activity is less than 1/5. Said strain 1A51 has been deposited at the Ministry of International Trade and Industry, Agency of Industrial Science and Technology, Bioengineering Industry Technology Institute as FERM P-14066.

[0016]

Because NeuGc production is reduced for the mutant strains of this invention, glycoproteins of low NeuGc content can be produced when they are used as host cells in recombinant gene techniques. That is, by introducing recombinant vectors having genes coding for glycoproteins into the mutant strains of this invention and culturing the transformants obtained, glycoproteins with low NeuGc content that do not contain the H-D antigen can be produced. Since the mutant strains of this invention here have the same properties as the usual CHO cells aside from the fact that NeuGc production is low, the exogenous gene introduction method, culture method, product glycoprotein isolation method, etc., used when said mutant strains are used as the host cells can be the same as the methods that were used with CHO cells in the past.

[0017]

For target glycoproteins, human-derived glycoproteins are preferable. For example, erythropoietin, tissue plasminogen activating factor, granulocyte colony-formation stimulating factor, urokinase, type B hepatitis vaccine, etc., can be cited. Vectors into which the genes coding for these glycoproteins are incorporated are not particularly restricted as long as they are vectors that can express those genes in CHO cells. Common stable expression vectors, pcD2, pL2neoSRαIII, pMIKHygB, pMKITNeo, pSV2bsr, pRC/CMV, pRC/RSC, pcDNA3, pMAM-neo, etc., can be cited. For vectors that are favorable for high expression using dhfr-cells, pAdD26SVp(A), p91023(B), pSVMdhfr, pSV2dhfr, etc., can be cited. Said recombinant vectors can be introduced into the mutant strains of this invention using, for example, competent cell methods, calcium phosphate coprecipitation methods, electroporation methods, DEAE-dextran methods, Lipofectin methods, etc.

[0018]

The target glycoproteins are produced by culturing the transformed cells obtained, and extracting and separating them from said cultured cells and/or culture solution. For the cultivation of the transformed cells, various natural and synthetic media are used. Media that do not contain NeuGc are preferable. Moreover, it is preferable that the medium contains carbon sources such as

sugars, alcohols, organic acid salts, etc.; nitrogen sources such as protein mixtures, amino acids, ammonium salts, etc.; and inorganic salts. Furthermore, the addition of vitamins and antibiotics corresponding to selective marker genes is desirable. If it is a vector for which expression can be controlled, a procedure for inducing gene expression must be added during cultivation. After cultivation, the culture solution and cultured cells are separated by centrifugation. When the glycoprotein accumulates in the cultured cells, after the cells are disrupted using, for example, freezing and thawing, ultrasound, a French press, enzyme treatment, homogenization, etc., the glycoprotein must be solubilized using, for example, EDTA, a surfactant, urea, guanidine hydrochloride, etc.

[0019]

By applying the culture solution or cultured cell extract obtained that contains the glycoprotein to various kinds of chromatographic columns, a purified glycoprotein can be obtained. Ion-exchange, affinity, gel filtration column chromatography, etc., can be used alone or in combination.

[0020]

The glycoproteins thus obtained do not contain the H-D antigen and are useful as pharmaceutical products, diagnostic drugs, etc.

[0021]

Application example

Next, this invention is explained in further detail citing an application example. But this invention is not restricted in any way by this application example.

[0022]

Application example

(1) CHO-K1 cell mutagenesis

Mutagenesis was performed by culturing CHO-K1 cells (Dainippon Pharmaceutical Co.) in medium [Ham's F-12 medium (Dainippon Pharmaceutical Co.) containing 10% fetal calf serum] containing 400 μg/mL EMS (ethylmethanesulfonate) at 33°C for 18 h.

[0023]

(2) Selection of cells with low amounts of GM3 expression by a complement-dependent cytotoxicity reaction using anti-GM3 antibodies

In flasks, CHO cells that were treated with a mutagen in (1) were cultured in Ham's F-12 medium containing 10% fetal calf serum at 37°C and in the presence of 5% CO_2 [culturing under these conditions will be called simply 'culturing' below]. These cells were dislodged by trypsin-EDTA treatment. After rinsing with Buffer Solution A [Ham's F-12 medium containing 20 mM HEPES (pH 7.3) and 10% fetal calf serum, same below), they were centrifuged for 2 min at 90 G. The supernatant was removed and 150 μL of anti-GM3 monoclonal antibodies (M2590, Cosmo Bio Co., 1 mg/mL) and 150 μL of rabbit complement (Dainippon Pharmaceutical Co.) were added to the cell pellet and reacted at 37°C for 1.5 h. After the reaction, they were rinsed 1 time with medium and cultured at 37°C in the presence of 5% CO_2. The above procedure was performed 2 times, and by culturing in 100 mm dishes, about 1000 colonies of cell strains thought to have low amounts of GM3 expressed were obtained from 2 million mutagen-treated CHO cells.

[0024]

(3) Detection of cells surface GM3 by indirect fluorescent antibody method

The cells obtained in (2) were cultured for 2 – 3 days on 35 mm dishes or lab dish chambers. The medium was removed and they were reacted with anti-GM3 monoclonal antibodies (10 μg/mL) in Buffer Solution A at 0°C for 30 min. The cells were rinsed 4 times with Buffer Solution A and were reacted with biotin-labeled anti-mouse IgM antibodies (Funakoshi Co., 2.5 μg/mL) in Buffer Solution A at 0°C for 30 min. The cells were rinsed 4 times with Buffer Solution A and reacted with SA-FITC (streptavidin-fluorescein isothiocyanate) complement (Cosmo Bio Co., 2.8 μg/mL) in Buffer Solution A at 0°C for 30 min. The cells were rinsed 4 times with PBS and FITC fluorescence was detected in PBS using the ACAS570 (Meridian Co.). As a result of this, expression of GM3 on the cell surface was reduced in 11 strains of the ca. 1000 strains obtained in (2). These 11 strains were cultured by the limiting dilution method and when the amount of cell surface GM3 was measured again by the above indirect fluorescent antibody method, GM3 expression was reduced in 9 strains.

[0025]

(4) In situ assay of enzymes in the glycolipid synthesis system

For 4 of the 9 strains obtained in (3), in situ sugar synthesis was studied by the following methods. Cells cultured in flasks were dislodged by trypsin-EDTA treatment and rinsed 3 times with PBS. After the cell membranes were damaged by freezing and thawing to facilitate permeation of glyconucleotide substrates into the cells, a reaction solution containing UDP-[^{14}C] galactose was added and reacted at 37°C for 30 min while shaking.

[0026]

Table 1 The composition of the reaction solution was as follows: 50 mM MES [2-(N-morpholino)ethanesulfonate (hydrate) buffer, pH 6.4, 1 mM NADH, 5 mM DTT (dithiothreitol), 5 mM $MnCl_2$, 2.5 mM $MgCl_2$, 22.2 μM UDP-[^{14}C] galactose (Amersham Co., 12.2 GBt/nmol)

[0027]

After completion of the reaction, the cell suspension was collected by centrifugation. After rinsing the precipitate 3 times with PBS, the cells were lyophilized. Labeled glycolipids were extracted with $CHCl_3$:MeOH (2:1), (1:1), (1:2) and analyzed by TLC. $CHCl_3$:MeOH:0.02% $CaCl_2 \cdot 2H_2O$ = 60:35:8 was used for the developing solvent. The radioisotope was detected with a BioImaging Analyzer BAS 2000 made by Fuji Photo Film (Co. Ltd.). The exposure was about 16 h.

[0028]

The results obtained are shown in Figure 1. From the TLC images of Figure 1, 2 bands corresponding to LacCer (Galβ1 → 4Glcβ1 → 1Cer) and 3 bands corresponding to GM3 were seen in the parent strain. In contrast to this, in Strain 1A51, there were 2 bands corresponding to LacCer as in the parent strain but there were 2 bands corresponding to GM3. The migration of the 2 upper bands of GM3 of the parent strain coincided with the 2 bands of GM3 of strain 1A51 and the migration of these 2 bands coincided with standard GM3 (sialic acid is NeuAc). It is known that when there is 1 kind of sialic acid molecule, 2 bands are generated because there are 2 kinds of Cer. Consequently, in CHO cells, there are 2 kinds of GM3 with differing sialic acid molecules. It was thought that in strain 1A51, however, one of these was missing.

[0029]

To confirm this, the reactions were performed by adding CMP-NeuAc (Sigma Co.) or CMP-NeuGc during the in situ assay of glycolipid synthesis. As a result of this, as shown in Figure 2, both the parent strain and strain 1A51 had only the upper 2 bands with CMP-NeuAc added to the reaction solution, and it was thought that the upper 2 were GM3 containing NeuAc as the sialic acid. When CMP-NeuGc was added to the reaction solution, the lower 2 bands became stronger, suggesting that the lower 2 bands were GM3 containing NeuGc as the sialic acid. Of these, strain 1A51 was thought to be a mutant strain in which NeuGc production was reduced.

[0030]

The CMP-NeuGc used in the above reactions was produced as follows. That is, CMP-NeuGc synthesis was performed according to the method of Herman et al. (J. Biol. Chem. 260;8838-8849 (1985)). To 200 μL of distilled water, 100 μL of 67 mM CTP, 100 μL of 22 mM NeuGc, 126 μL of 50 mM $MnCl_2$, 75 μL of 50 mM DTT, 400 μL of 200 mM Tris-HCl (pH 7.4), and 20 μL of recombinant CMP-sialic acid synthase (Cosmo Bio Co.) (180 mU) were added and reacted at 37°C for 1 h. After the reaction, 9 mL of ethanol were added and the CMP-NeuGc was precipitated.

[0031]

(5) Sialic acid analysis

Sialic acid analysis of the CHO cells and strain 1A51 was performed according to the method of Hara et al. (Anal. Biochem. 179:162-166 (1989)). Cells cultured in flasks were dislodged by trypsin-EDTA treatment and rinsed 3 times with PBS. The cells were suspended in 200 μL of aqueous 2M acetic acid and hydrolysis of the sugar chains was performed by treating at 80°C for 3 h. After the reaction, the cell residue was removed by centrifugation. 200 μL of DMB (1,2-diamino-4,5-methylenedioxybenzene) solution (7 mM DMB, 1.4M acetic acid, 0.75M 2-mercaptoethanol, 18 mM sodium bisulfite) were added and reacted at 50°C for 2.5 h to fluorescent-label the sialic acid. The fluorescent-labeled sialic acid was quantified using HPLC. A Hitachi F-1050 fluorescence detector connected to a BioRad Co. Model 2700 System was used. The excitation wavelength was 373 nm, and the emission wavelength was 448 nm. For the column, ToSo [Company's] TSK gel ODS-120T (250 x 4.6 mm) was used. For the eluent, acetonitrile:methanol:water = 9:7:84 was used. The flow rate was 0.9 mL/min. As a result of this, the main sialic acids present in CHO cells were NeuAc and NeuGc only. NeuGc content was 14.2% in the parent strain and 5% in strain 1A51.

[0032]

(6) Measurement of CMP-NeuAc hydroxylase activity

Since NeuGc is generated by hydroxylation of CMP-NeuAc by CMP-NeuAc hydroxylase, CMP-NeuAc hydroxylase activities of CHO cells and strain 1A51 were measured to study whether or not the cause of reduced NeuGc content in Strain 1A51 was due to lack of this enzyme. CMP-NeuAc hydroxylase activity of CHO cells was measured according to the method of Kawano et al. (Glycoconjugate J. 10:109-115 (1993)). Cells cultured in flasks were dislodged by trypsin-EDTA treatment and rinsed 3 times with PBS. A suspension in 10 mM Tris-HCL (pH 7.5) after the cell membranes had been damaged by freezing and thawing was used as the enzyme

source. A reaction solution containing CMP-NeuAc was added and reacted at 37°C for 60 min while shaking.

[0033]

Table 2. The composition of the reaction solution (50 μL) was as follows.

10 mM Tris-HCl (pH 7.5)

5 μM Cytochrome b5

12 μg NADH-dependent cytochrome b5 reductase

40 μM CMP-NeuAc

1 mM DTT

0.7 mM NADH

[0034]

After completion of the reaction, 200 μL of cold ethanol were added. After leaving undisturbed at 0°C for 15 min, it was centrifuged at 15000 rpm for 5 min to sediment the protein. Quantitation of the enzymatically produced CMP-NeuGc contained in these supernatants was performed using HPLC. A Model 1706 UV/VIS monitor was connected to a BioRad Co. Model 2700 System and the absorbance at 271 nm was measured. For the column, ToSo TSK gel [column] ODS-80TM (250 x 4.6 mm) was used. For the eluent, 50 mM monobasic ammonium phosphate was used. The flow rate was 0.5 mL/min. Protein quantitation was performed using BioRad Company's protein assay kit with bovine serum albumin as the standard protein.

[0035]

As a result of this, as shown in Figure 3, the CMP-NeuAc hydroxylase activity of strain 1A51 was less than 1/5 of the parent strain. Consequently, strain 1A51 is a strain that lacks CMP-NeuAc hydroxylase, and for this reason it was found to be a cell strain in which the cell content of NeuGc is low (production is reduced).

[0036]

Effect of the invention

Since NeuGc production is reduced for the mutant CHO cell strains of this invention, a glycoprotein produced by recombinant gene technology that uses these as host cells contains almost no NeuGc. Consequently, the mutant strains of this invention are useful as host cells for producing glycoproteins that do not have H-D antigenicity.

Brief description of the figures

Figure 1 is a figure showing in situ assay results (TLC) for glycolipid synthesis system enzymes of CHO-K1 cells (parent strain) and strain 1A51.

Figure 2 is a figures showing the effects of adding CMP-NeuAc or CMP-NeuGc on in situ assays of glycolipid synthesis system enzymes of CHO-K1 cells (parent strain) and strain 1A51.

Figure 3 is a figure showing CMP-NeuAc hydroxylase activities of CHO-K1 cells (parent strain) and strain 1A51.

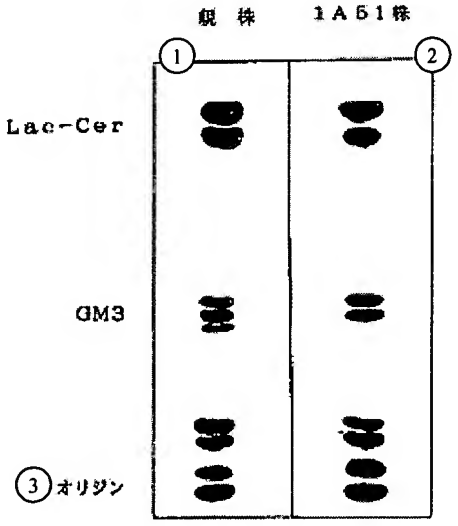

Figure 1

Key: 1 Parent strain
2 Strain 1A51
3 Origin

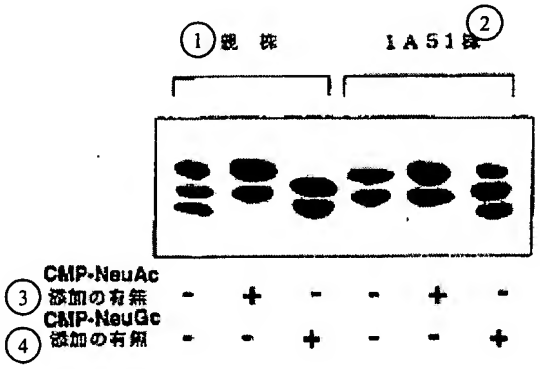

Figure 2

Key: 1 Parent strain
2 Strain 1A51
3 CMP-NeuAc added or not
4 CMP-NeuGc added or not

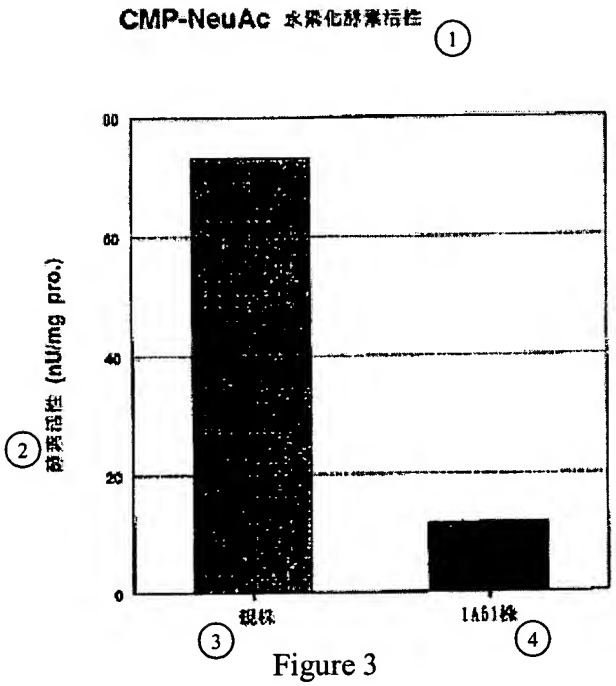

Figure 3

Key:
1 CMP-NeuAc hydroxylase activity
2 Enzyme activity (nU/mg protein)
3 Parent strain
4 Strain 1A51